

The Tyrosyl Free Radical in Ribonucleotide Reductase

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The enzyme, ribonucleotide reductase, catalyses the formation of deoxyribonucleotides from ribonucleotides, a reaction essential for DNA synthesis in all living cells. The *Escherichia coli* ribonucleotide reductase, which is the prototype of all known eukaryotic and virus-coded enzymes, consists of two non-identical subunits, proteins B1 and B2. The B2 subunit contains an antiferromagnetically coupled pair of ferric ions and a stable tyrosyl free radical. EPR studies show that the tyrosyl radical, formed by loss of an electron, has its unpaired spin density delocalized in the aromatic ring of tyrosine. Effects of iron-radical interaction indicate a relatively close proximity between the iron center and the radical. The EPR signal of the radical can be studied directly in frozen packed cells of *E. coli* or mammalian origin, if the cells are made to overproduce ribonucleotide reductase.

The hypothetical role of the tyrosyl free radical in the enzymatic reaction is not yet elucidated, except in the reaction with the inhibiting substrate analogue 2'-azido-CDP. In this case, the normal tyrosyl radical is destroyed with concomitant appearance of a 2'-azido-CDP-localized radical intermediate. Attempts at spin trapping of radical reaction intermediates have turned out negative.

In *E. coli* the activity of ribonucleotide reductase may be regulated by enzymatic activities that interconvert a nonradical containing form and the fully active protein B2. In synchronized mammalian cells, however, the cell cycle variation of ribonucleotide reductase, studied by EPR, was shown to be due to *de novo* protein synthesis.

Inhibitors of ribonucleotide reductase are of medical interest because of their ability to control DNA synthesis. One example is hydroxyurea, used in cancer therapy, which selectively destroys the tyrosyl free radical.

Introduction

Ribonucleotide reductase is the only known enzyme that carries a stable free radical in its resting state. The radical can be observed in intact cells as well as in the isolated, homogeneous enzyme. Ribonucleotide reductase is an essential enzyme of all living cells. It catalyses the first step on the biochemical pathway leading to DNA synthesis by reducing ribonucleotides to their corresponding deoxyribonucleotides (Fig. 1). General reviews on the enzyme have been published recently (1-4). This review is more specifically devoted to the properties and functions of the free radical of the enzyme.

The most extensively studied ribonucleotide reductase is the one isolated from the bacterium *Escherichia coli*. It is the prototype of all known eukaryotic and virus-coded ribonucleotide reductases (Fig. 2). It consists of two nonidentical subunits, proteins B1 and B2, each of which are enzymatically inactive alone. The active enzyme is formed by a 1:1 complex of proteins B1

and B2, in which B1 contributes redoxactive sulfhydryls and B2 contributes the unique tyrosyl radical stabilized by an adjacent binuclear iron center. Protein B1 contains two nearly identical polypeptide chains of 87 kDalton each. Protein B2 contains two identical polypeptide chains of 43 kDalton each. The amino acid sequences of the B1 and B2 polypeptide chains were recently deduced from the nucleotide sequence of the corresponding *E. coli* genes (5). Binding of the substrate molecules, ribonucleoside diphosphates, occurs at the B1 subunit. This subunit also contains two different classes of binding sites for the allosteric effector molecules, nucleoside triphosphates. One class of binding sites regulates the specificity of the enzyme to reduce any of the four possible ribonucleotide substrates. The other class of binding sites regulates the overall activity of the enzyme, which can be efficiently turned off in a feed back regulated manner by the nucleotide dATP. The allosteric regulation has been studied in considerable detail and variations on the general theme occur with enzymes from different origins (1,4,6).

In some bacteria and algae, other prototypes of ribonucleotide reductase are found (4). One type, first isolated from the bacterium *Lactobacillus leichmannii*,

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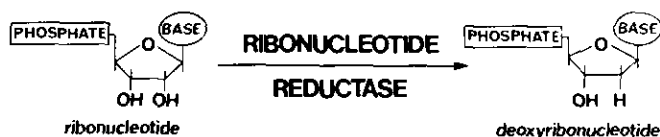


FIGURE 1. Enzymatic reaction catalyzed by ribonucleotide reductase.

and extensively studied, reduces ribonucleoside triphosphates. It consists of a single polypeptide chain and a vitamin B₁₂ cofactor. Other types of recently isolated and characterized enzymes are oligomeric and utilize B₁₂ or divalent metal ions like Mn²⁺ as cofactors. It seems as if microorganisms display a variety of evolutionary solutions to the chemically complex reaction of reducing a secondary alcohol. Of these only the *E. coli* variant has been selected to serve in the eukaryotic kingdom. The simplest common denominator of all types of ribonucleotide reductases seems to be redoxactive sulfhydryls and a metal-containing cofactor.

The electrons needed for the reduction of ribonucleotides are transported through a redox chain utilizing NADPH as the ultimate reductant. Two hydrogen donor systems are known (?). One is the thioredoxin system composed of thioredoxin and thioredoxin reductase. The other is the glutaredoxin system composed of glutaredoxin, glutathione and glutathione reductase. The mutual importance of these two systems for ribonucleotide reduction is presently not clear.

The remarkable similarity in *E. coli* and eukaryotic ribonucleotide reductases is prominently manifested in the active site of the metal-containing subunit, which in all cases studied so far harbors a stabilized unpaired electron in a tyrosine side chain of its polypeptide backbone. Not only is the localization of the radical to a

tyrosine residue specific, also the geometry of the tyrosyl radical is very similar in ribonucleotide reductases from *E. coli*, bacteriophage T4, mouse cells and pseudorabies virus (8–11). For the first three of these enzymes it has also been shown that the tyrosyl radical coexists with an adjacent binuclear iron center (12–14).

Another remarkable similarity between species is evident in the primary structures of ribonucleotide reductases as deduced from their DNA sequences in *E. coli* (5), herpes simplex virus (15,16), Epstein Barr virus (17), and the surface clam *Spisula solidissima* (18) (Fig. 3). Although the N-terminal and C-terminal parts of the B2-equivalent subunits show a low degree of homology, three short internal regions are strikingly conserved (19). Such distant evolutionary relationships have the advantage that most invariant residues are likely to be functionally important. In Figure 3 we want to emphasize residue Tyr 122 as a candidate for radical stabilization. This is the only conserved tyrosine residue besides Tyr 356, which can be excluded because B2 lacking this C-terminal part still carries the tyrosyl radical (20). Close to Tyr 122 one finds the possible iron ligands His 118 and Glu 115. In one of the other conserved areas further candidates for iron ligation are found in His 241 and Asp 237 or Glu 238. The definite proof of these residues being directly involved in the active site of protein B2 must await the determination of its tertiary structure. Recently the crystallographic parameters were obtained for high resolution crystals of protein B2 from *E. coli* (21).

The Tyrosyl Radical

Spectroscopic Properties and Structure

More than 10 years ago it was observed that purified preparations of ribonucleotide reductase from *E. coli* gave a characteristic EPR signal, which could not be explained by the known presence of iron in the sample. The EPR spectrum (Fig. 4a), most easily observed at low temperatures, was tentatively assigned to a free radical (22). The magnitude of the EPR signal was found to be correlated with the activity of the enzyme. Also associated with the EPR signal was a sharp peak at 410 nm in the optical absorption spectrum of the enzyme.

Due to the small amounts of ribonucleotide reductase in wild type *E. coli* cells, more detailed structural studies had to await the development of a bacterial strain which could overproduce the enzyme. This strain, which is lysogenic for a recombinant bacteriophage, carries the structural genes for the bacterial ribonucleotide reductase attached to the λ genome (23). Heat induction of the lysogenic strain gives about 50 times more of ribonucleotide reductase than what is produced in wild type cells. This makes it possible to study the ribonucleotide reductase-specific EPR signal directly in *E. coli* cell suspensions without previous enzyme purification, since the specific EPR signal greatly dominates over all other cellular contributions in the free radical region of the spectrum.

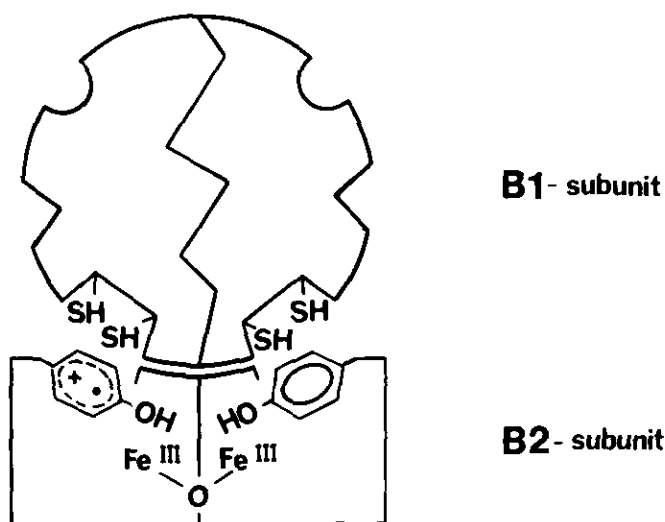


FIGURE 2. Model of the *Escherichia coli* ribonucleotide reductase. The two types of allosteric binding sites are indicated in the B1 subunit.

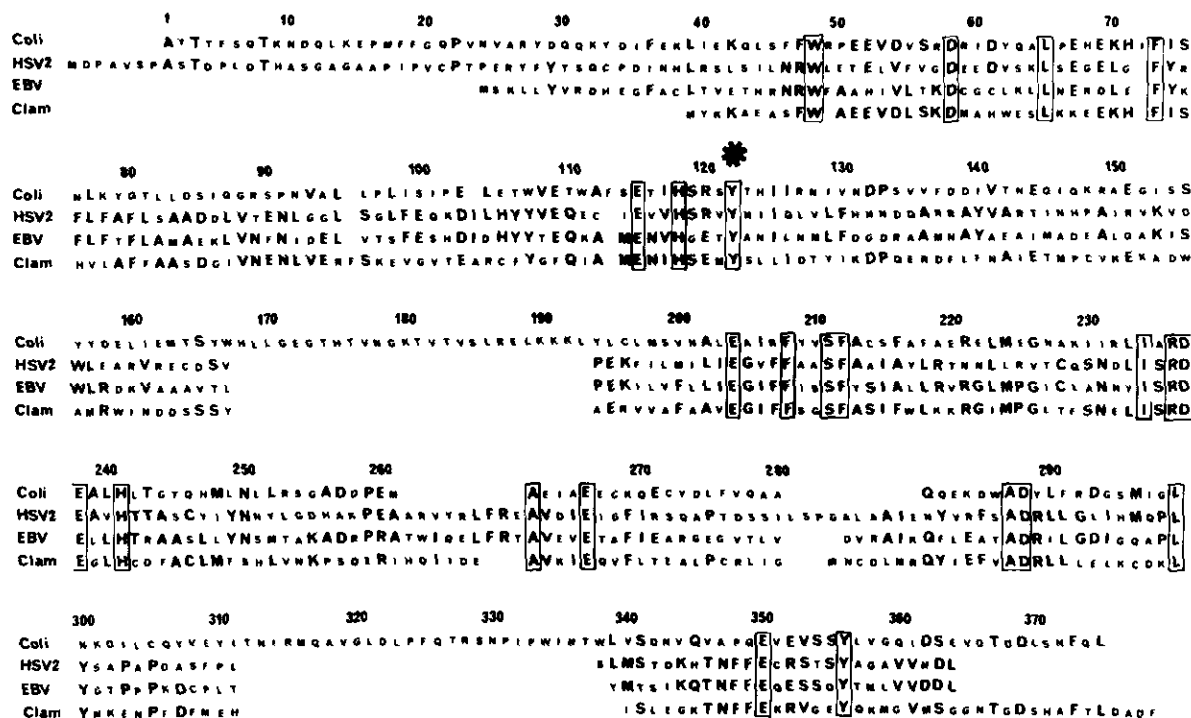


FIGURE 3. Amino acid sequence alignment of *E. coli* protein B2 (5), herpes simplex virus 38K protein (16), Epstein-Barr virus 34K protein (17) and *Spisula solidissima* 42K protein (18). Structurally equivalent residues are in big capitals. Invariant residues of all four sequences are within boxes. An asterisk indicates the proposed site of the tyrosyl radical (19). The numbering refers to the *E. coli* sequence.

Taking advantage of the experimental possibilities opened by this overproducing bacterial strain, isotope substitutions were performed by adding labeled compounds to the bacterial growth medium (8,24). When deuterium substitutes for hydrogen in a free radical, the hyperfine pattern caused by protons changes in a characteristic way due to the different magnetic properties of H and D nuclei. The D nucleus with spin quantum number $I = 1$ has a magnetic moment which is only 15% of that of the H nucleus with $I = 1/2$. An EPR doublet hyperfine splitting arising from H therefore, after D exchange, becomes a narrower triplet splitting, often coalescing into a single unresolved line. An effect of this was directly observed in cells grown in a medium based on 93% D_2O . The major hyperfine doublet splitting for cell suspensions grown in a H_2O medium (Fig. 4a) collapsed into an unresolved singlet line in a D_2O medium.

Further isotope substitutions localized the radical to a tyrosine residue. Figure 5 shows the effects in the EPR spectra of specifically deuterated tyrosines included in the growth media and incorporated into the enzyme: Deuterium at positions 2, 6, and α gives only a slight decrease in the linewidth of an otherwise unchanged EPR spectrum (Fig. 5a; for tyrosine numbering see Fig. 6). Deuterium at positions 3 and 5 causes the 7 G triplet structure to disappear (Fig. 5b). Deuterium at positions β causes the major 19 G doublet splitting to disappear (Fig. 5c). Therefore it was concluded that the unpaired spin density is delocalized in

the aromatic ring of tyrosine (8). By using equations relating hyperfine coupling constants and spin densities in a delocalized aromatic radical (25), spin densities were estimated as shown in Figure 6 (9). The spin density at C_1 gives rise to the 19 G hyperfine coupling to one of the methylene protons. The second methylene proton in this case does not give any observable hyperfine coupling, for reasons that will be discussed later. However, the inequality of the two methylene protons indicates that, at least at low temperature, the aromatic ring is in a locked conformation relative to the methylene group.

The spin density distribution of the enzyme radical is similar to that of oxidized tyrosine (26–28). Tyrosine is an easily oxidizable amino acid and the oxidized tyrosyl radicals have been observed in several studies using 1-electron oxidation (26,29,30). Recently, Sealy et al. made an EPR study of the temperature dependence of the EPR parameters of chemically oxidized tyrosine at alkaline pH. Also in this case the results showed a basic inequivalence of the two β methylene protons even above room temperature (31).

The presence of a stable oxidized tyrosyl radical seems to be a common feature in all iron-containing ribonucleotide reductases studied so far. Figure 4 shows the EPR spectra of other reductases of widely different origins: bacteriophage T4-induced enzyme in *E. coli* cells (9), mammalian enzyme (10,32) and pseudorabies-induced enzyme in mammalian cells (11). The T4-induced enzyme and the mammalian enzyme have been

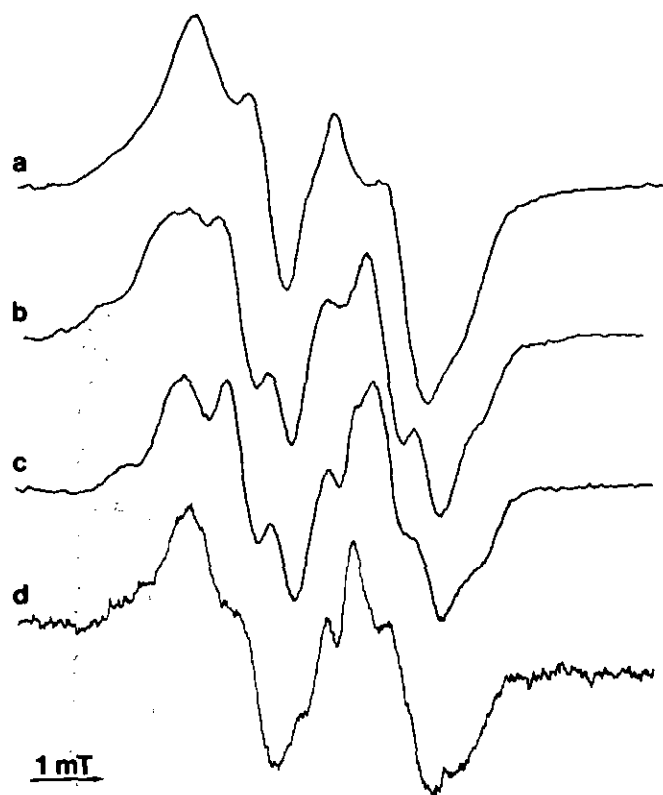


FIGURE 4. EPR spectra of tyrosine free radicals of ribonucleotide reductase of various origins: (a) *E. coli* cells overproducing ribonucleotide reductase at 32°K. The wild type cell background was negligible. (b) Purified ribonucleotide reductase from bacteriophage T4-infected *E. coli* at 77°K. (c) Hydroxyurea-resistant mouse 3T6 cells at 32°K. A suitable background of wild type cells has been subtracted. (d) Pseudorabies virus-infected mouse L cells at 32°K. A background spectrum of mock-infected mouse L-cells has been subtracted.

duced enzyme and the mammalian enzyme have been shown to contain two nonidentical subunits, formally corresponding to the B1 and B2 proteins in the *E. coli* enzyme. In the T4-induced enzyme the two subunits are bound together more tightly than the subunits of *E. coli* (33). In the mammalian case, the subunits are called M1 and M2. Protein M1 is a dimer of molecular weight 170 kDalton (34,35). Protein M2 has recently been purified to homogeneity and is a dimer of molecular weight 88 kDalton (14). For the T4 and mammalian enzymes, isotope substitution experiments similar to those already described for *E. coli* were used to assign the radical to a tyrosine residue (9,10). The dominating doublet hyperfine splitting remains similar in all four cases, indicating a similar immobilized conformation at low temperature for the four enzymes.

The minor differences between the hyperfine patterns exhibited at low temperatures by the four different enzymes in Figure 4 are most likely due to small geometrical differences in the conformations of the immobilized tyrosine residues harboring the free radicals. The magnitude of the hyperfine coupling a_H to each of the β methylene protons, caused by the unpaired spin

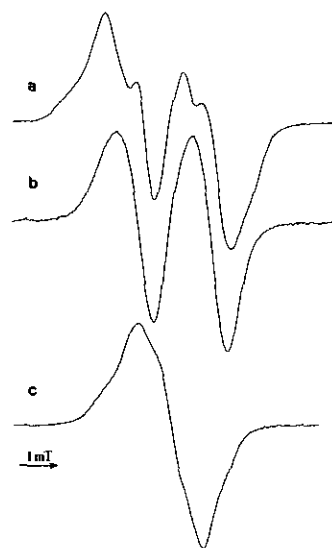


FIGURE 5. EPR spectra at 77°K of cell suspensions of *E. coli* KK546 which overproduce ribonucleotide reductase. The bacteria were grown in the presence of (a) [2,6, α -D₃]-tyrosine, (b) [3,5-D₂]-tyrosine, and (c) [β , β -D₂]-tyrosine (8).

density at C_1 (Fig. 6), is critically dependent on the angle θ between the orbital axis of the radical (perpendicular to the plane of the aromatic ring) and the C_β -H bond projected perpendicular to the C_1 - C_β bond. The following relationship holds: $a_H = \rho(B_0 + B_2 \cos^2 \theta)$, where ρ is the spin density at C_1 , $B_0 \sim 0$ G, and $B_2 \sim 50$ G (9,25,36).

Applying this relation to the hyperfine couplings evaluated from the spectra of the T4-induced and native *E. coli* radicals, we obtained the following angles θ for the two methylene protons (9): T4-induced, 10° and 130° from two measurable couplings; *E. coli*, approximately 0° and 120°, since only one coupling can be measured.

Another possibility for the variability of the different radical EPR spectra could be different distributions on different conformational states already present in each sample (31). Although this possibility cannot be excluded as at least a partial explanation for the variability, we consider it less likely since each spectrum is

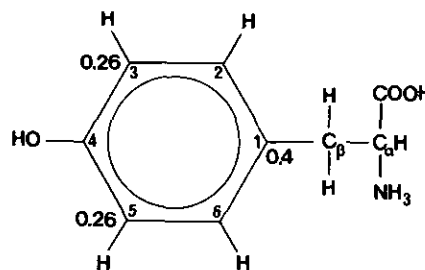


FIGURE 6. Estimated spin density distribution in the tyrosyl free radical of *E. coli* and bacteriophage T4-induced ribonucleotide reductase (2,8,9).

completely reproducible regardless of state of purification of the enzyme and velocity of freezing.

The optical spectra of the *E. coli* protein B2 and the mammalian protein M2 radicals are shown in Figure 7. The radical spectra are obtained as the differences between those of native and nonradical-containing B2 or M2 (37,12,14). Nonradical-containing protein was obtained through treatment with hydroxyurea which specifically destroys the ribonucleotide reductase radicals (see below). Both spectra are similar to the light absorption spectrum of a chemically induced 2,4,6-tritertiary butyl phenoxy radical, shown in Figure 7c (12,38,39). This is also further proof that the enzyme radicals are formed through loss of an electron. However, whether the enzyme radicals also formally are phenoxy radicals or have hydrogen or a ligand bound to O₄ is in our opinion an open question.

Stability

It is unusual to find a stable free radical as an integral part of an enzyme. The stability of the protein B2 radical is closely connected to the presence of the iron center of the enzyme. The iron center of protein B2 has been characterized by Mössbauer spectroscopy (37), light absorption (12,37), magnetic susceptibility (12), and resonance Raman techniques (13,40). It is composed of a pair of high spin ($S = 5/2$) ferric ions, antiferromagnetically coupled through a μ -oxo bridge. When the iron is chelated out of the protein, the tyrosyl radical is also lost (37). The radical and enzyme activity may then be restored, if ferrous iron is presented to the protein in the presence of oxygen (12,37). In the mammalian enzyme, recent light absorption studies on purified protein M2 showed a similar iron center (14).

Hydroxyurea and hydroxylamine are radical scavengers which specifically destroy the tyrosyl radical in the ribonucleotide reductases (22,37). The radical scavengers probably act through 1-electron donation (41). In protein B2 the tyrosyl radical may be restored and the enzyme reactivated by iron chelation and subsequent addition of ferrous iron in the presence of oxygen. However, it was recently demonstrated that activation of nonradical-containing B2 is a pH-dependent reaction, which occurs spontaneously in the presence of dithiothreitol, oxygen, and Mg^{2+} above pH 8.0 (P. Reichard, personal communication).

In protein M2 the radical is less stable than in B2 and is easily lost, e.g., in enzyme purification procedures. The M2 radical is also easily restored in the presence of a reducing agent (dithiothreitol), oxygen, and iron (42), without previous removal of iron through chelating agents.

Radical Content and Localization

Early on, Ehrenberg and Reichard observed a linear correlation between specific activity and radical content of protein B2 (22). Our best preparations show a stoi-

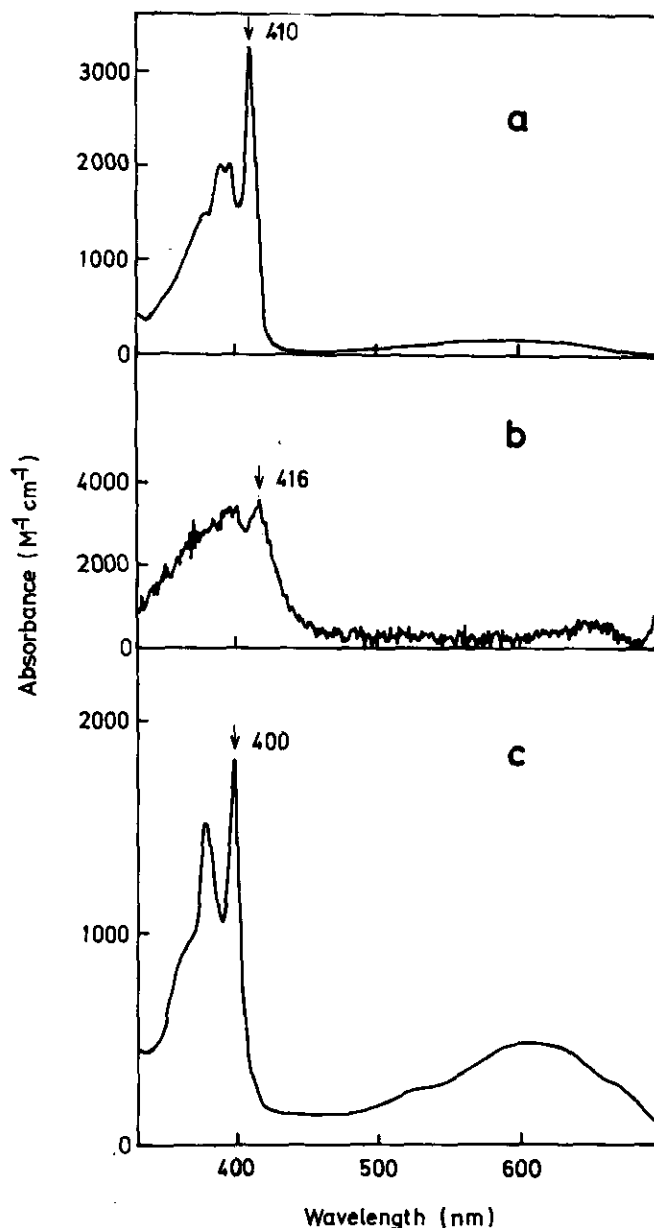


FIGURE 7. Light absorption spectra of the tyrosyl radicals of (a) *E. coli* protein B2, and (b) mouse protein M2. The spectra were obtained as differences between native and nonradical-containing proteins, (12,14). (c) Light absorption of the model radical 2,4,6-tritertiary butyl phenoxy radical in hexane replotted from Land et al. (38). The arrows indicate the positions of the sharp long-wavelength light absorption peaks.

chiometry of 1 tyrosyl radical per dimeric iron center of B2 (12). Because each B2 protein consists of two identical polypeptide chains, each B2 polypeptide dimer most likely harbors 1 tyrosyl radical. The possibility of a mixed population of protein B2 with radical contents of 0, 1, and 2 is not likely, because the same average radical content of approximately 1 has repeatedly been obtained in native preparations and in chelated and reconstituted preparations.

Recently we encountered another form of protein B2 in the overproducing cells (20,43). This form arises from

a limited proteolysis of the normal B2 ($\beta\beta$) dimers to give a B2 ($\beta'\beta'$) dimer, which has lost 29 C-terminal amino acid residues. B2 ($\beta'\beta'$) contains a normal iron center and a normal tyrosyl radical but has no enzymatic activity due to lack of binding to the B1 subunit. A hybrid dimer B2 ($\beta\beta'$) can be obtained in 50% yield from a stoichiometric mixture of normal B2 ($\beta\beta$) dimers and defective B2 ($\beta'\beta'$) dimers, which have been carried through a reconstitution cycle of iron chelation and reactivation. All three possible B2 dimers ($\beta\beta$, $\beta\beta'$, and $\beta'\beta'$) can be separated by ion exchange chromatography. It was possible to study the localization of the tyrosyl radical within the $\beta\beta'$ dimer by starting the reconstitution experiment described above from deuterated B2 ($\beta\beta$) and protonated B2 ($\beta'\beta'$). Consequently, every full-length β -polypeptide was deuterated and every digested β' -polypeptide was protonated so that the mixed dimers $\beta\beta'$ must always incorporate one protonated and one deuterated polypeptide chain. All three types of dimers contained approximately 1 radical per dimer and in the B2 ($\beta\beta'$) dimers we observed a mixed EPR signal consisting of 58% protonated doublet and 42% deuterated singlet contribution. This implies a fully random generation of the tyrosyl radical at either polypeptide chain of the dimer during the reconstitution reaction, a situation which closely resembles a "half-site" reactivity feature as is frequently found in allosterically regulated enzymes (44). It probably also implies that protein B2 can only serve to reduce one substrate molecule at a time, albeit the B1 subunit contains two fully active substrate binding sites. One may in fact consider protein B2 as a high molecular weight cofactor for protein B1, a further support for the formal similarity between the *E. coli* and *L. leichmannii* enzymes (4).

Iron-Radical Interaction

A striking difference between the *E. coli* protein B2 and mammalian protein M2 radicals is apparent in their different EPR microwave saturation behavior. At 77°K, the EPR signal of the M2 radical is extremely difficult to saturate with available microwave powers (~100 mW), whereas the B2 radical shows microwave saturation effects above 0.3 mW.

Towards higher temperatures, the EPR spectra of the ribonucleotide reductases are considerably broadened and lose their low temperature hyperfine structure (compare Fig. 8a with Fig. 4a). For the *E. coli* and mammalian enzymes, a detailed study of the temperature dependence of EPR signal lineshapes and microwave saturation was recently conducted (45,46). For the free radicals of both B2 and M2, the unusual line broadening of the EPR signal and the difficulty to achieve saturation at higher temperatures may be explained by magnetic interaction between the free radical and the antiferromagnetic iron center. The latter exhibits an increasing effective magnetic moment at increasing temperatures (12). In protein B2, the theory of magnetic dipolar interaction was found to give a satisfactory ex-

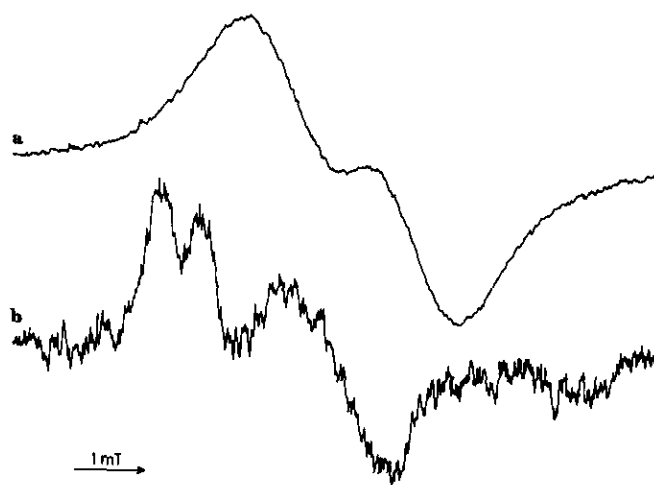


FIGURE 8. EPR spectra at room temperature of (a) the tyrosyl radical of protein B2 at a protein concentration of 112 μ M, and (b) the azido-CDP substrate radical after 5 min reaction in an incubation mixture containing 20 μ M of the *E. coli* ribonucleotide reductase (see text). The spectra were recorded in a flat cell using a TE-mode cavity. The microwave power was 20mW and the modulation amplitude was 0.2 mT (59).

planation for the results, which imply a rather close proximity between iron center and radical. In protein M2, the results are better explained by magnetic exchange interaction. Relatively minor sterical differences in the iron center/radical geometry could explain why one or the other magnetic interaction mechanism dominates in a given case.

Reaction Mechanism

Hypothetical Role of the Tyrosyl Radical

The enzymatic reaction which is catalyzed by ribonucleotide reductase is formally a reduction of the secondary alcohol at 2'-C in the ribose ring to a hydrocarbon with retention of configuration at this carbon. The reduction of nucleoside diphosphate substrates occurs at the expense of redoxactive sulfhydryls of protein B1 (47). Although the fully active B2 subunit with its tyrosyl radical is needed for catalysis, we have not so far observed any changes in the EPR signal or the optical spectrum of B2 during the catalytic process (48). There are, however, several indirect lines of evidence for the involvement of the tyrosyl radical in the catalytic process. These studies deal with K_{cat} suicidal substrate analogs, such as 2'-halogenated or 2'-azido-substituted nucleoside diphosphates (49-52).

A few years ago, Stubbe et al. demonstrated that specifically 3'-tritiated substrates were selected against with an isotope effect of approximately 3 (53,54). This supports their hypothesis that cleavage of the 3'-carbon-hydrogen bond is a primary step in ribonucleotide reduction. The 3'-hydrogen is most likely abstracted by

the tyrosyl radical of B2 to generate a 3'-nucleotide radical.

A less significant selection against 3'-tritium occurred with 2'-halogenated substrate analogs which specifically inhibits the B1 subunit. Stubbe et al. proposed a detailed mechanism for this suicidal reaction (54). The mechanism involves, besides the 3'-nucleotide radical, a radical cation intermediate which rapidly may decompose into a reactive 3'-ketone. Recently several of the hypothesized intermediates were observed (55). It was also shown that 2'-fluoro analogs can partition between a normal reduction to produce 2'-deoxy NDP and inactivation of B1. Reactions involving normal substrate are thus very likely to follow the same intermediary steps at least in the outset of the reaction and probably also involve a radical cation intermediate.

The suicidal reaction with azido-substituted analogs specifically destroys the tyrosyl radical of the B2 subunit. This K_{cat} inactivation involves a new radical intermediate which can be studied by EPR spectroscopy (50). Its characteristics are a large (25 G) triplet further split by a smaller (6 G) doublet (Fig. 8b). We proposed a sugar-localized neutral nitrogen-centered radical with an additional hyperfine coupling to a hydrogen. With specifically ^{15}N -labeled azido-UDP it was indeed corroborated that the new EPR species is localized to one of the azido-nitrogens (56), but demonstration of a hydrogen coupling has been negative for the ribose hydrogens at carbon 1' through 4' (56,57). Taken together, the results with 2'-halogenated and 2'-azido-substituted substrate analogs as well as the isotope effect at the 3'-C of the ribose ring are fully compatible with a radical cation mechanism as outlined in Figure 9.

In the mammalian ribonucleotide reductase prepared from calf thymus the M2 radical is labile and needs oxygen and a reducing agent to be continuously regenerated. The half-life of the radical under anaerobic conditions was found to be of the order of 10 min (42). The continuous presence of oxygen is also needed during the catalytic action. However, there is no specific consumption of oxygen in the catalytic cycle, since the initial reaction rate was approximately unchanged under anaerobic conditions compared to aerobic conditions (42).

Experiments were also performed with the *E. coli* ribonucleotide reductase to determine a possible oxygen requirement during catalysis (58). The EPR signal was monitored at 77°K after aerobic or anaerobic incubation of an enzyme reaction mixture at 25°C in the time interval 0–30 min. Samples were also tested for enzymatic activity during the same time interval. To ensure absolute anaerobicity, in one experiment, a system with addition of glucose oxidase, glucose and catalase was used. It was not possible in any experiment to demonstrate a requirement for oxygen in the *E. coli* system even after 3000 turnovers (58).

Spin-Trapping Experiments

In order to try to reveal the nature of the hypothetical substrate radical, spin trapping experiments were performed at room temperature (59). Three different spin traps were used, namely 5,5-dimethylpyrroline-*N*-oxide (DMPO), phenyl-*tert*-butylnitron (PBN), and *tert*-nitrobutane (MNP). Reaction conditions were equimolar concentrations of proteins B1 and B2 in a mixture containing the appropriate effector for assay conditions at pH 7.6 (60) but with dithiothreitol as a reducing agent instead of the thioredoxin system. Both normal substrate, CDP, and the substrate analog azido-CDP were used. No substrate radical could be trapped with any of the spin traps. Likewise, it was not possible to trap the azido-CDP radical. However, it could be observed that the azido radical spectrum at room temperature (Fig. 8b) is in principle the same as that obtained at 77°K, showing the same anisotropy of the major nitrogen hyperfine splitting (50). This is in contrast to the loss of structure observed for the B2 radical at higher temperatures (Figs. 8a and 4a). Thus the substrate analog radical appears to be in a locked conformation at the active site of the enzyme even at room temperature. Furthermore the radical is not close enough to the binuclear iron center for its EPR spectrum to be broadened at higher temperatures.

Since the EPR signal from protein B2 disappears upon addition of the radical scavenger hydroxyurea, spin-trapping experiments with MNP were also per-

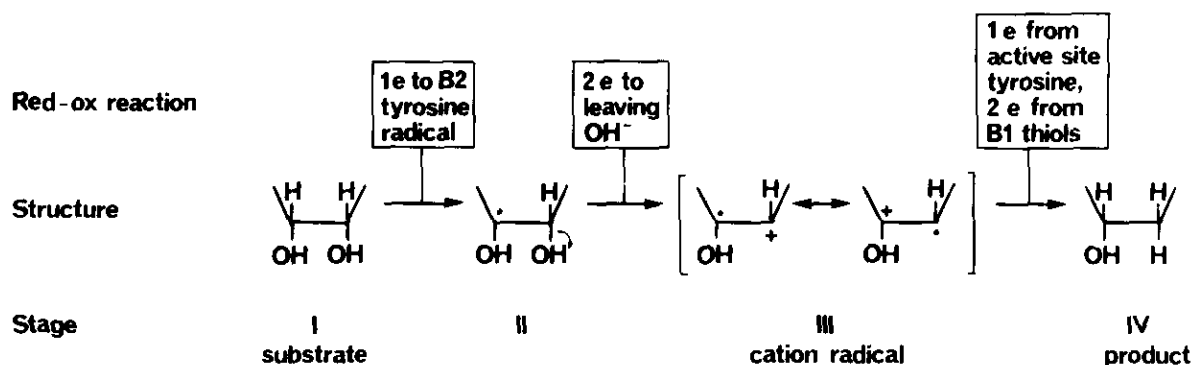


FIGURE 9. Proposed reaction mechanism for ribonucleotide reductase in its reduction of ribonucleoside diphosphates (50,53).

formed during the hydroxyurea reaction. However, no evidence for any trapped radical was found.

Finally, reconstitution of protein B2 from apoprotein was performed with a reactivation mixture containing Mohr's salt (37) and with DMPO as a spin trap. The results were immediate reconstitution with no radical trapped.

To summarize the spin-trapping experiments, we conclude that the *E. coli* ribonucleotide reductase keeps its tyrosyl radical in a rather rigid and protected conformation. We also infer that the substrate radical (as observed with azido-CDP and postulated with normal substrate) must be inaccessible for the spin traps at the conditions used, probably due to the geometry of the active site and/or the short lifetime of the substrate radical.

Similarities with Reaction Mechanism of Ribonucleotide Reductase from *L. leichmannii*

In discussing the reaction mechanism of *E. coli* ribonucleotide reductase it is worthwhile to consider the remarkable similarities to the reaction mechanism of the *L. leichmannii* enzyme. Isotope labeling studies indicate that also this enzyme cleaves the 3'-C-H bond of the ribose moiety (61). A radical cation mechanism for the *L. leichmannii* enzyme is particularly intriguing, since the adenosylcobalamin cofactor in this case has been shown to undergo a homolytic scission of the cobalt-deoxyadenosine bond during catalysis (62). The function of adenosylcobalamin may be as a radical chain initiator. Its hypothetical role could be to facilitate the formation of a protein localized but exceedingly short-lived equivalent of the tyrosyl radical (4,61).

In Vivo Regulation of the Tyrosyl Radical

The B2-equivalent subunit of any ribonucleotide reductase has to undergo post-translational modification in order to become a fully active subunit with iron and tyrosyl radical from the nascent ApoB2 form. As pointed out earlier, *in vitro* reconstitution of the *E. coli* ApoB2 form occurs spontaneously in the presence of ferrous iron and oxygen, constituents which are certainly available in anaerobically grown *E. coli* cells. It is presently not known which oxidizing agent might take the role of oxygen in anaerobically grown cells.

Protein B2 of *E. coli*

The tyrosyl radical of B2 is remarkably stable during storage conditions. For instance, a sample of 1 mM B2 in deuterated phosphate buffer retained 80% of its free-radical content after storage at 4°C for about one month (58). Slow but measurable half-lives can, however, be observed at ambient temperatures and, e.g., during ca-

talysis (23,37). This leads to an inactive form of B2 lacking the tyrosyl radical. Similar forms may also exist in an *in vivo* situation.

Extracts from *E. coli* were recently shown to contain activities that interconvert the nonradical-containing and the fully active forms of protein B2 (3,63). The inactivating system has not yet been studied in any depth. The radical introducing system on the other hand has been studied and turns out to be complex. It involves several enzyme activities, a few of which are flavine dependent, and it requires the presence of a nicotinamide cofactor, reducing agent, oxygen and Mg^{2+} (64). This enzyme-catalyzed reactivation proceeds well at pH 7.0 in contrast to the chemical activation mentioned above which requires a pH above 8.0.

Another radical-containing enzyme requiring an activating system is pyruvate formate-lyase from *E. coli* which is induced during anaerobic cultivation of the bacteria. Knappe et al. recently showed that the activating reaction in this case resulted in the introduction of a free radical into the inactive form of the enzyme (65). The assignment of its doublet EPR spectrum has not been clarified but preliminary experiments indicate that it is not localized to a tyrosine residue (66). Pyruvate formate-lyase is the second example of an enzyme having a stable free radical as an integral part. Since EPR spectroscopy is not a routine technique to characterize enzymes, the future might reveal yet more catalytically essential free radicals of this kind.

Protein M2 and the Mammalian Cell Cycle

For the mammalian ribonucleotide reductase a study of the cell cycle regulation of the enzyme has been carried out in mouse mammary tumor cells (67). The cells were selected for overproduction of protein M2 by continuous culturing over a period of one year in stepwise increasing concentrations of the radical scavenger hydroxyurea. The M2 radical can be determined quantitatively "*in vivo*" by EPR measurements of such frozen, packed cells. When the cells are grown in a medium containing deuterated tyrosine instead of normal tyrosine, the characteristic collapse of the EPR hyperfine doublet spectrum into a singlet is observed. This fact allows discrimination between *de novo* synthesis of protein M2 versus possible reactivation of "old" inactivated nonradical-containing protein upon change of cell medium from containing normal to deuterated tyrosine. A 3 to 7-fold increase in M2 radical was observed when synchronized mouse cells passed from the G₁ to the S phase of the cell cycle and this increase was due to *de novo* synthesis. In contrast it was observed that after hydroxyurea inactivation and removal of the drug, the cells were able to rapidly regenerate the tyrosyl radical from "old" nonradical-containing M2 protein.

Medical Aspects

The ability to control DNA synthesis is of considerable medical interest. A selective inhibition of DNA

synthesis is desired, e.g., in clinical applications such as restriction of tumor growth or virus replication. In this context ribonucleotide reductase occupies a crucial stand at the intersection between RNA and DNA precursor biosynthesis. Specific inhibitors of ribonucleotide reductase like hydroxyurea and azidocytidine have been used as agents in cancer therapy (4).

The accessibility of the tyrosyl radical of *E. coli* ribonucleotide reductase has been thoroughly explored by use of a series of hydroxyurea analogs (41). These compounds can donate an electron to the tyrosyl radical. The reaction rate with B2 is essentially proportional to the reaction rate between the same analog and the radical compound Fremy's salt. Very bulky side chains on chemically reactive analogs however are completely inactive with the tyrosyl radical of B2. From such experiments the tyrosyl radical of B2 could be assigned to a pocket in the protein of approximately 4×6 Å.

In the mammalian enzyme the localization of the tyrosyl radical and the iron center must be more exposed, since chemically reactive and bulky compounds such as polyhydroxybenzohydroxamic acids exert potent inhibition at concentrations as low as 10 μ M. The same compounds are virtually inactive with *E. coli* B2, most likely due to steric hinderance. Polyhydroxybenzohydroxamic acids are presently explored as antiproliferative drugs because of their low general toxicity to mammalian cells.

One of the most potent inhibitors known for the mammalian ribonucleotide reductase is the iron chelate of 1-formylisoquinoline thiosemicarbazone (IQ-1). It has been shown that the target of this drug is the tyrosyl radical of protein M2 (68). After removal of the drug, regeneration of the radical could be achieved during normal assay conditions. A closer analysis showed that the reducing agent dithiothreitol under aerobic conditions was responsible for the regeneration, i.e., the same conditions as after hydroxyurea inactivation were needed. It was also found that oxygen was needed for the tyrosyl radical destruction by IQ-1. It was therefore postulated that the iron chelate of IQ-1 destroys the M2 tyrosyl radical by a one-electron redox process with participation of iron and oxygen.

Further exploration of specific inhibitors for ribonucleotide reductases seems promising, especially since it has been demonstrated that several medium-sized virus genomes (pseudo-rabies, herpes simplex and Epstein-Barr) code for virus-specific ribonucleotide reductase of the common prototype but with some distinctly virus-specific characteristics (11,16,17).

Up to now, no enzymes induced by eukaryotic viruses have been produced in amounts sufficient to make meaningful quantitative comparative inhibition studies. In the *E. coli* case, which is the only one known, it is however clear that the bacteriophage T4-induced enzyme is considerably more sensitive to hydroxyurea than is the host enzyme (9,41). If this is a more general phenomenon, one may look for other radical scavengers which are more potent against viral enzymes than against their host enzymes.

Ribonucleotide reductase is one of approximately a handful of enzymes specific for active DNA synthesis in living organisms. Due to its unique tyrosyl radical and delicate allosteric regulation it comprises a very promising target for dedicated inhibition of DNA synthesis.

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